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Review

Triacylglycerol and wax ester-accumulating machinery in prokaryotes

Héctor M. Alvarez

Centro Regional de Investigación y Desarrollo Científico Tecnológico (CRIDECIT), Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, CIT-CHUBUT, CONICET, Km 4-Ciudad Universitaria 9000, Comodoro Rivadavia, Chubut, Argentina

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ABSTRACT

Gram negative bacteria as well as Gram positive actinobacteria possess the ability to accumulate variable amounts of wax esters (WE) and/or triacylglycerols (TAG) under nitrogen limiting conditions. In recent years many advances have been made to obtain insight into neutral lipid biosynthesis and accumulation in prokaryotes. The clinical and industrial relevance of bacterial WE/TAG significantly promoted basic and applied research in this field. The recent integrated omic studies as well as the functional characterization of diverse genes are contributing to unravel the composition of the WE/TAG-accumulating machinery in bacteria. This will be a valuable data for designing new drugs against bacteria with clinical importance, such as *Mycobacterium tuberculosis*, or for transferring and optimizing lipid accumulation in bacterial hosts naturally unable to produce such lipids, such as *Escherichia coli*. In this article, recent investigations addressing WE/TAG biosynthesis and storage in prokaryotes are presented. A comprehensive view of the current knowledge on the different genes/proteins involved in WE/TAG biosynthesis is included.

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1. Introduction

Most bacteria are able to survive and thrive in environments with fluctuating nutritional conditions. Moreover, bacterial cells also interact with multiple stress factors that simultaneously occur in natural environments. The production of neutral lipids, such as wax esters (WE) and triacylglycerols (TAG), may be part of the complex strategic survival mechanisms evolved by some prokaryotes, which allow them to colonize and thrive in natural environments. These lipids are convenient storage compounds for carbon and energy, which can be utilized for cell survival in energy-poor environments. Since the carbon atoms of acyl moieties of TAG and WE are in their most reductive form; the degradation of these biomolecules produces a maximum yield of energy in comparison to other storage compounds produced by bacteria, such as glycogen and polyhydroxyalkanoates [1]. The energy obtained by the slow

E-mail address: halvarez@unpata.edu.ar

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mobilization of stored lipids may provide cells of energetic autonomy and a temporal independence from the environment and contribute to cell survival when they do not have access to energy resources in the environment. Lipid stored by bacteria may be important not only for their energy potential but also as a reservoir of metabolic water under desiccation conditions, since fatty acid oxidation releases large amounts of metabolic water [2]. In addition, storage lipids possess other important functions in cells, such as the regulation of the fatty acid composition of membrane lipids, as a sink for reducing equivalents and physiological active and potentially toxic metabolic intermediates for balancing the metabolism under environmental fluctuating conditions, as precursor source for biosynthesis of essential lipids, among other possible functions [3].

The biosynthesis and accumulation of TAG and/or WE are stimulated when an excess of a carbon source is available and the nitrogen source is limiting [4,5]. These special conditions are frequently found in soil and marine environments. The ability to accumulate storage lipids demands the presence of a genetic and enzymatic endowment in the microorganism and the capability for maintaining the balance of precursors and reducing equivalents since the lipid accumulation is an energy-expensive process, which compete with cellular growth. The process of neutral lipid accumulation and their involved components have been well studied in eukaryotic organisms, such as plants and yeasts [6,7]. The pioneer studies on WE and TAG accumulation in prokaryotes were mainly performed in members of *Acinetobacter* [4], *Mycobacterium* [8], *Streptomyces* [9] and *Rhodococcus* [10] genera. The important role of TAG in the pathogenesis of *Mycobacterium tuberculosis,* and the relationship of TAG metabolism with antibiotic biosynthesis by *Streptomyces coelicolor* have stimulated the basic research on such lipids in those microorganisms. On the other hand, members of *Acinetobacter* and *Rhodococcus* genera, such as *Acinetobacter baylyi* ADP1 and *Rhodococcus opacus* PD630 have been used as models for deciphering different aspects on WE/TAG biosynthesis and accumulation. More recently, other bacteria with the ability to produce WE and/or TAG have emerged as model organisms for different studies in this field, including *Marinobacter hydrocarbonoclasticus* [11], *Alcanivorax borkumensis* [12] and *Rhodococcus jostii* [13]. The potential application of such neutral lipid-producing microorganisms as a source of single cell oil useful for the production of biofuels or other derived industrial products, promoted further studies which contributed with our understanding of the process. Single cell oils are lipids extracted from microorganisms, which could serve as alternative oil sources for the production of biofuels with similar efficiency as petroleum diesel. The use of microorganisms for lipid production provides some advantage over agricultural sources with regards to the enormous variability of fatty acid composition depending on the carbon source used for cultivation of cells, and the better accessibility of microorganisms to genetic and metabolic engineering. Current research efforts are being focused on the biochemistry and genetics of oil-accumulating bacteria for designing a scalable and commercially viable oil-producing system from inexpensive feedstocks. In this context, the application of omic approaches as well as the functional identification and characterization of key genes/proteins from model bacteria, enabled significant advances in the fundamental knowledge on WE/TAG metabolism. This review article provides a comprehensive view on the composition of the WE/TAG-accumulating machinery necessary for supporting biosynthesis and accumulation of such lipids in prokaryotes.

2. Synthesis and accumulation of WE/TAG by bacteria

TAG as well as WE are synthesized by a diversity of bacteria. However, there are some qualitative and quantitative differences in their accumulation profiles. The synthesis and accumulation of TAG and WE have been reported for Gram negative hydrocarbon-degrading bacteria belonging to *Acinetobacter*, *Marinobacter*, *Thalassolituus* and *Alcanivorax* genera [4,14,15]. These microorganisms are able to produce TAG and WE during cultivation of cells on acetate, pyruvate or hexadecane as sole carbon sources. In general, those cells accumulate low amounts of neutral lipids during growth under nitrogen limiting conditions. However, some strains such as *A. baylyi* ADP1 and *A. borkumensis* SK2, are able to accumulate about 10–20% of cellular dry weight (CDW) of neutral lipids [15,16]. Usually, there are differences regarding the production of TAG and WE depending on the strain. In the case of *A. borkumensis* SK2 and *Alcanivorax jadensis* T9 TAG are the main neutral lipids accumulated, whereas *A. baylyi* ADP1 and *Marinobacter hydrocarbonoclasticus* SP17 and DSM 8798 accumulate mainly WE [11,15].

On the other hand, the accumulation of neutral lipids is a common feature of Gram positive actinobacteria, such as those belonging to *Streptomyces*, *Gordonia*, *Mycobacterium*, *Rhodococcus* and *Nocardia* [8,9,17]. Among them, the members of mycolic acid-containing actinobacteria seem to be the TAG-accumulating specialists. Most of these microorganisms accumulate exclusively TAG when cells are cultivated on non-related carbon sources, such as gluconate or glucose [1]. Large amounts of TAG and markedly more than Gram negative bacteria are usually accumulated by actinobacterial cells during growth on different carbon sources. *Rhodococcus opacus* PD630, which represents the paradigm of TAG-accumulating bacteria, is able to produce up to 75% (CDW) of TAG during cultivation of cells on gluconate under nitrogen limiting conditions [10]. *R. opacus* PD630 produced WE in addition to TAG, only during growth of cells on *n*-alkanes or phenylalkanes as sole carbon sources [10,18]. Altogether, the combination of the substrate used as carbon source for cell growth and the metabolism of the bacterial strain determine the type of neutral lipid accumulated by bacterial cells.

3. Key acyltransferase enzymes for TAG and WE synthesis in bacteria

The synthesis of TAG and WE in prokaryotes depends on the presence of a CoA-dependent acyltransferase enzyme known as wax ester synthase/diacylglycerol acyltransferase (WS/DGAT). This enzyme can exhibit simultaneously both, acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WS) and diacylglycerol acyltransferase (DGAT) activities (Fig. 1). The first prokaryotic WS/DGAT was reported for *A. baylyi* ADP1 by Kalscheuer and Steinbüchel [16]. Later, several WS/DGATs were identified, cloned and characterized in different strains of *Marinobacter*, *Alcanivorax*, *Mycobacterium*, *Streptomyces* and *Rhodococcus* [11,15,19–22]. In general, WS/DGATs are promiscuous enzymes that accept a broad diversity of acyl-CoA substrates for esterification of DAG or long-chain fatty alcohols for the synthesis of TAG or WE, respectively [16,23] (Figs. 1 and 2). This property confers to bacterial cells the ability to produce TAG and/or WE depending on which intermediates are present in the cellular metabolism. The presence of WS/DGAT enzymes seems to be a key feature that differentiates bacteria capable for synthesizing TAG or WE to those unable to produce such lipids. The heterologous expression of the WS/DGAT enzyme from *A. baylyi* ADP1 or that from *Streptomyces colelicolor* in *Escherichia coli* conferred the ability to produce low amounts of TAG in this bacterial host which is not naturally able to synthesize these neutral lipids [24,25].

In general, Gram negative TAG/WE-synthesizing bacteria possess low number of *ws/dgat* genes in their genomes (1–3 copies) [23]. A similar situation was observed in members of *Streptomyces* genus, such as *S. coelicolor* (3 *ws/dgat's*) [20] and *Streptomyces avermitilis* (1 *ws/dgat*) [26]. In contrast, mycolic acid-containing actinobacteria

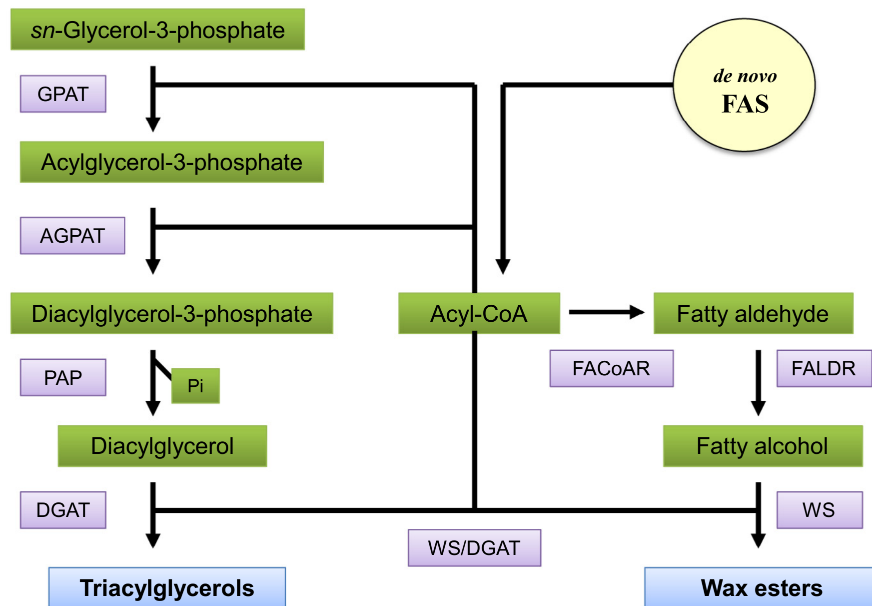

Fig. 1. Biosynthesis pathways of TAG and WE in prokaryotes. Reactions of the Kennedy pathway for the synthesis of TAG from glycerol-3-phospahte and acyl-CoA (in the left) and reactions involved in the synthesis of WE from acyl-CoA (in the right). Abbreviations: WS, wax ester synthase; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate O-acyltransferase; AGPAT, acyl-glycerol 3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; FALDR, fatty aldehyde reductase; FACoAR, fatty acyl-CoA reductase; FAS, fatty acid synthase.

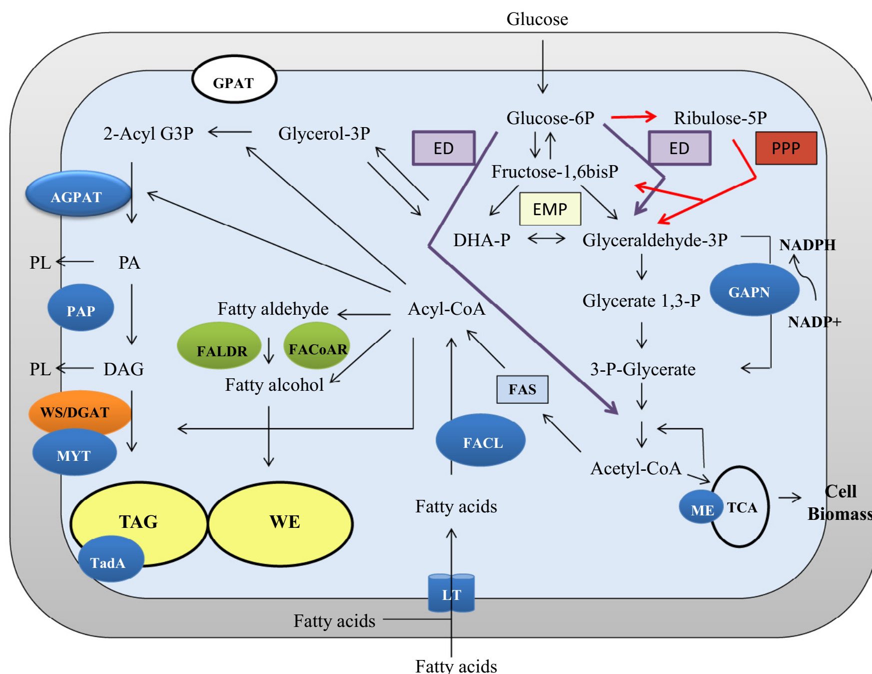

Fig. 2. Genes/proteins involved in the WE/TAG-accumulating machinery experimentally identified in prokaryotes. The proteins identified uniquely in Gram negative bacteria are shown in green ovals; whereas those identified only in Gram positive actinobacteria are shown in blue ovals. In the orange oval are included WS/DGAT enzymes, which have been identified in both, Gram negative, as well as Gram positive bacteria, with the ability to accumulate WE/TAG. Abbreviations: EMP; Embden-Meyerhoff pathway; ED, Entner–Duodoroff pathway; PPP, Pentose Phosphate pathway; FAS, fatty acid synthase; PL, phospholipids; WE, wax esters; TAG, triacylglycerols; WS, wax ester synthase; DGAT, diacylglycerol acyltransferase; GAPN, non-phosphorylative glyceraldehyde dehydrogenase enzyme; ME, malic enzyme; GPAT, glycerol-3-phosphate O-acyltransferase; AGPAT, acyl-glycerol 3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; MYT, mycolylacyltransferase enzyme; TadA, structural protein of lipid bodies; LT, lipid transporter protein; FACL, fatty acyl-CoA synthetase; FALDR, fatty aldehyde reductase; FACoAR, fatty acyl-CoA reductase.

possess a high abundance of *ws/dgat* genes in their genomes (5–17 isoenzymes) [22], with the exception of *Rhodococcus fascians* F7, which contains only two putative WS/DGATs [27]. The number of *ws/dgat* genes found in bacterial genomes seems to be a strain dependent feature. In this context, the oleaginous *R. opacus* PD630 and *R. jostii* RHA1 possess 17 and 15 genes coding for WS/DGAT enzymes in their genomes, respectively [13,22]. The sequences of these WS/DGAT isoenzymes exhibit a high diversity in both strains. The occurrence of large number and diversity of WS/DGAT isoenzymes in bacteria may permit cells to incorporate a diversity of fatty

acids into neutral lipids or to regulate differentially lipid accumulation according to the environmental conditions. In general, all WS/DGAT enzymes of Gram negative- and also Gram positive bacteria exhibit different substrate selectivities and activities as revealed diverse *in vitro* and *in vivo* studies. For more details on the structure and specificity of bacterial WS/DGAT enzymes, readers can read some review and original articles [23,28,29] or articles on specific lipid-accumulating strains or WS/DGAT enzymes. The substrate specificity and activity of each WS/DGAT enzyme may be in direct relationship to the usually available carbon sources present in the natural environment of each microorganism, and the available precursors formed by its metabolic network.

The presence of WS/DGAT enzymes enables cells to synthesize TAG and/or WE; however, the accumulation of such lipids seems to be the result of the metabolic dynamics and regulation in each microorganism. The accumulation of TAG or WE is a carbon- and energy-expensive process, which competes for precursors and reducing equivalents with anabolic pathways involved in cell growth. Beyond the presence of WS/DGAT enzymes, lipid-accumulating bacteria need to possess an efficient metabolic network capable of producing the required amounts of precursors, reducing equivalents and energy from the available carbon sources, and a regulatory programme that permit cells maintaining a high carbon flux toward the lipid biosynthesis pathways.

4. Routes that feed precursors for neutral lipid biosynthesis

The key metabolic intermediates feeding lipid biosynthesis are pyruvate, acetyl-CoA and glycerol-3-phosphate. Pyruvate is the end product of glycolysis, which is one of the switch points for carbon flux distribution within the central metabolism. This metabolic intermediate can serve as precursor for sugar phosphate synthesis through the gluconeogenesis or can be used to replenish TCA cycle intermediates that are bled off for anabolic processes (anaplerotic reactions) [30]. In addition, pyruvate can be decarboxylated to acetyl-CoA, which is the basic precursor for fatty acid, and eventually fatty alcohol biosynthesis. Bacterial cells must possess an efficient mechanism for producing an available pool of acetyl-CoA for supporting WE or TAG accumulation. Diverse pathways may contribute to the formation of acetyl-CoA pool in bacteria. Sugar degradation pathways, principally the Entner–Doudoroff (ED) and pentose phosphate (PPP) pathways, might be the major route of carbon flux to fatty acid biosynthesis, since they provide not only the necessary intermediates such as pyruvate, but also reducing equivalents as NADPH for feeding the *de novo* fatty acid biosynthesis pathway. In this context, sugars usually support good growth and neutral lipid accumulation principally in actinobacteria [10,31]. Recently, Hollinshead et al. [32] reported that ED pathway is highly active in *R. opacus* PD630 under aerobic conditions. The catabolism of amino acids may serve as an alternative source for acetyl-CoA and propionyl-CoA production, both of which can be used for fatty acid biosynthesis [33,34]. In addition, several reactions of the amino acid degradation generate NADPH, which can be used for lipid biosynthesis.

Another key precursor which has to be available in cells for TAG biosynthesis and accumulation, but not for the synthesis of WE, is glycerol-3-phosphate (G3P). This intermediate is also needed for the biosynthesis of membrane phospholipids; thus, bacteria that are unable to produce TAG are still able to produce G3P. For this reason, TAG-accumulating bacteria must be able to generate an additional pool of G3P or switch the distribution of this intermediate from phospholipid to TAG biosynthesis pathway, to some extent (Fig. 2). In this context, such microorganisms must possess fine metabolic and regulatory mechanisms to control G3P availability in cells.

On the other hand, the *de novo* fatty acid biosynthesis is a high NADPH- and ATP-consuming process, since the synthesis of one molecule of palmitic acid requires 8 molecules of acetyl-CoA, 14 molecules of NADPH and 7 molecules of ATP. Usually, bacterial cells must arrest growth and replication and shift their metabolism, carbon flux, energy and reducing equivalents to lipid biosynthetic pathways.

Altogether, the ability to synthesize and accumulate TAG and/or WE by prokaryotes demands a specific metabolic and regulatory network for activating the necessary metabolic reactions that provide precursors, energy and NADPH for lipid synthesis. The metabolic and regulatory configuration (i.e. the differential arrangement of metabolic reactions and regulatory circuits as instrinsic characteristics of each microorganism) together with the occurrence of key specific genes/enzymes seems to define the potential of bacterial cells to synthesize and accumulate WE and TAG.

5. Triacylglycerol- and wax ester-biosynthetic machinery in Gram negative bacteria

Gram negative bacteria belonging to *Acinetobacter*, *Marinobacter*, and *Alcanivora*x are the most studied microorganisms regarding neutral lipid accumulation, principally WE. Among them, *A. baylyi* ADP1 is the main model representative in the field. Biosynthesis of WE by strain ADP1 involves three enzymatic steps; firstly, an acyl-CoA is reduced to a corresponding long-chain aldehyde by a NADPH dependent fatty acyl-CoA reductase (called Acr1) [35], the resulting fatty aldehyde is further reduced to a corresponding fatty alcohol by a yet uncharacterized aldehyde reductase, and finally the fatty alcohol is esterified with a fatty acyl-CoA by the well characterized bifunctional enzyme WS/DGAT, resulting in the formation of a WE molecule [36] (Table 1, Figs. 1 and 2). Kalscheuer and Steinbüchel [16] identified and characterized in detail the WS/DGAT enzyme (known as AtfA) of *A. baylyi* ADP1, which mediates WE and TAG biosynthesis and accumulation. Interestingly, a residual trace amount of TAG was present in *atfA*-deleted mutants, suggesting the existence of an alternative low-rate pathway for TAG synthesis in this bacterium [16].

Rontani et al. [14] reported the ability of *Marinobacter hydrocarbonoclasticus* DSM 8798 to synthesize an isoprenoid WE during growth on phytol as sole carbon source under nitrogen limiting conditions. Later, the biosynthesis of isoprenoid WE was investigated at molecular level by Holtzapple and Schmidt-Dannert [11]. The authors identified an isoprenoid specific CoA synthetase (Acs2) and two isoprenoid WS/DGAT enzymes (WS1 and WS2), which were involved in the biosynthesis of WE by strain DSM 8798 (Table 1). Among three putative WS/DGAT enzymes cloned from *M. hydrocarbonoclasticus*, WS1 and WS2 were capable for synthesizing isoprenoid WE, whereas WS3 did not exhibit activity with any of the acyl or isoprenoid substrates used in the study [11]. The authors proposed that WS3 may be not involved in WE or TAG synthesis or be nonfunctional. *Marinobacter aqualeolei* VT8 is also able to synthesize medium and long chain-fatty acyl and fatty alcohols for the biosynthesis of WE from unrelated carbon sources [37,38]. It has been proposed that *M. aqualeolei* and *M. hydrocarbonoclasticus* belong to the same species [28,39]. Fatty alcohols are produced in *M. aqualeolei* via a two step reduction of either acyl-CoA or acyl-ACP to the respective fatty alcohol via an intermediate fatty aldehyde (Fig. 1) [37,38,40]. These authors demonstrated the existence of two fatty acyl-CoA reductases (termed FarA and AcrB) (Maqu_2220 and Maqu_2507) in *M. aqualeolei* VT8, with the ability to reduce not only fatty aldehydes, but also acyl-CoAs and acyl-ACPs to fatty alcohols in an NADPH-dependent manner (Table 1). Interestingly, these novel bacterial enzymes from strain VT8 catalyze the four-electron reduction of fatty acyl-CoA substrates to the corresponding fatty alcohol, in contrast to other reports for *A. bayili* ADP1 FACoAR enzyme that only reduce fatty acyl-CoA by two electrons to the fatty aldehyde [38]. It is noteworthy that from more than 1800 microbial

Table 1
Enzymes/proteins of the lipid-accumulating machinery identified and characterized in TAG- and WE-accumulating bacteria. Abbreviations: WE, wax esters; TAG, triacylglycerols; DAG, diacylglycerols; WS, wax ester synthase; DGAT, diacylglycerol acyltransferase; AGPAT, acyl-glycerol 3-phosphate acyltransferase; PAP2, phosphatidic acid phosphatase 2.

Strain and protein ID	Protein name	Protein name	Accession number	Function	Source
Acinetobacter baylyi ADP1					
ACIAD3383	Acr1	Fatty acyl-CoA reductase	YP_047869	Reduction of fatty acyl-CoA to fatty aldehyde	Reiser and Somerville (1997)
ACIAD0832	AtfA	WS/DGAT	YP_045555.1	WE and TAG synthesis	Kalscheuer and Steinbüchel (2003)
Marinobacter hydrocarbonoclasticus DSM 8798					
ABO21017	Acs2	Isoprenoid-CoA synthetase	EF219373	Activation of isoprenoid fatty acids	Holtzapple and Schmidt-Dannert (2007)
ABO21020	WS1	WS	EF219376	WE synthesis	Holtzapple and Schmidt-Dannert (2007)
ABO21021	WS2	WS	EF219377	WE synthesis	Holtzapple and Schmidt-Dannert (2007)
Marinobacter aqualeolei VT8					
Maqu_2220	FarA	Fatty acyl-CoA reductase	YP_959486.1	Reduction of fatty acyl-CoA or fatty acyl-ACP to fatty alcohol	Hofvander et al. (2011) Lenneman et al. (2013)
Maqu_2507	AcrB	Fatty acyl-CoA reductase	YP_959769.1	Reduction of fatty acyl-CoA to fatty alcohol	Lenneman et al. (2013) Willis et al. (2011)
Maqu_168	Ma1	WS	YP_957462.1	WE synthesis	Lenneman et al. (2013)
Maqu_3067	Ma2	WS	YP_960328.1	WE synthesis	Lenneman et al. (2013)
Alcanivorax borkumensis SK2					
ABO_2742	AtfA1	WS/DGAT	YP_694462.1	WE and TAG synthesis	Kalscheuer et al. (2007)
ABO_1804	AtfA2	WS/DGAT	YP_693524.1	WE and TAG synthesis	Kalscheuer et al. (2007)
Streptomyces coelicolor A3(2)					
SCO2951	–	NAD⁺-dependent malic enzyme	CAB72197	Decarboxylation of malate to pyruvate	Rodriguez et al. (2012)
SCO5261	–	NADP⁺-dependent malic enzyme	CAC05958	Decarboxylation of malate to pyruvate	Rodriguez et al. (2012)
SCO1102	Lppα	PAP2	NP_625396.1	Synthesis of DAG from phosphatidic acid	Comba et al. (2013)
SCO1753	Lppβ	PAP2	NP_626025.1	Synthesis of DAG from phosphatidic acid	Comba et al. (2013)
SCO0958	–	WS/DGAT	NP_625255.1	TAG synthesis (major contribution)	Arabolaza et al. (2008)
SCO1280	–	WS/DGAT	NP_625567.1	TAG synthesis (minor contribution)	Arabolaza et al. (2008)
SCO2386	FasR	Transcriptional regulator	NP_626632.1	Activation of fatty acid synthesis	Arabolaza et al. (2010)
Streptomyces avermitilis MA-4680					
SAV7256	–	WS/DGAT	NP_828432	TAG synthesis	Kaddor et al. (2009)
Mycobacterium tuberculosis H37Rv					
Rv3130c	Tgs1	WS/DGAT	CCP45940.1	TAG synthesis	Daniel et al. (2011)
Rv3804c	–	Mycolyltransferase Ag85A	CCP46633	Mycolic acid synthesis and TAG biosynthesis	Elamin et al. (2011)
Rv1206	FACL6	Acyl-CoA synthetase	CCP43962.1	Activation of fatty acids	Daniel et al. (2015)
Rv3391	Fcr1	Fatty acyl-CoA reductase	CCP46212.1	Reduction of fatty acyl-CoA to fatty alcohol	Sirakova et al. (2012)
Rv1543	Fcr2	Fatty acyl-CoA reductase	CCP44307.1	Reduction of fatty acyl-CoA to fatty alcohol	Sirakova et al. (2012)
Mycobacterium bovis BCG					
BCG1489c	–	AGPAT	BCG1489c	Formation of phosphatidic acid	Low et al. (2010)
BCG1169c	–	–	BCG1169c	Unknown function	Low et al. (2010)
BCG1721	–	Lipase and ACSL	BCG1721	Hydrolysis of TAG and activation of free fatty acids to fatty acyl-CoA	Low et al. (2010)
BCG3153c	Tgs1	WS/DGAT	BCG3153c	TAG synthesis	Low et al. (2010)
BCG3794c	Tgs2	WS/DGAT	BCG3794c	TAG synthesis	Low et al. (2010)
Rhodococcus opacus PD630					
OPAG_03892	TadD	Non-phosphorylative glyceraldehyde dehydrogenase	EHI47090	Oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate	MacEachran and Sinskey (2013)
OPAG_00658	TadA	Structural protein	AHK33470	TAG body-associated protein	MacEachran et al. (2010)
OPAG_07257	Atf1	WS/DGAT	EHI42943	TAG synthesis	Alvarez et al. (2008)
OPAG_00138	Atf2	WS/DGAT	EHI41112	TAG synthesis	Hernández et al. (2013)
Rhodococcus jostii RHA1					
RHA1_RS27545	Ltp1	ABC Transporter	YP_705582	Importer of fatty acids	Villalba and Alvarez (2014)
RHA1_RS00400	PAP2	Phosphatidic acid phosphatase	YP_700069.1	Synthesis of DAG from phosphatidic acid	Hernández et al., 2015
RHA1_RS10270	TadA	Structural protein	ABG93911	TAG body-associated protein	Ding et al. (2012)

genomes that were screened for the occurrence of Maqu_2220 homologs, only five genomes belonging to gammaproteobacteria contained ORF's with significant homology. All bacterial genomes containing putative FACoAR enzymes belonged to marine strains of *Marinobacter*, *Hahella*, *Oceanobacter* and *Desulfatibacillum* genera, suggesting that the ability to reduce fatty acyl residues to fatty alcohols for the synthesis of WE is a frequent feature in marine bacteria [37].

Lenneman et al. [40] reported a certain redundancy of several of the enzymes involved in WE synthesis by *M. aqualeolei* VT8, in contrast to other WE-accumulating bacteria, such as *A. baylyi* ADP1, which possesses only a single enzyme for each WE biosynthesis reaction. Strain VT8 possesses four homologs for the WS/DGAT enzymes and two alternative FACoAR enzymes (AcrB and FarA) to reduce fatty aldehydes and activated fatty acids to fatty alcohols [40] (Table 1). Single and double deleted FACoAR mutants analyses revealed that either AcrB or FarA enzyme is capable for supporting WE synthesis independent of one another. Only trace amounts of WE were produced in the absence of both genes. However, FarA seems to be the major enzyme contributing to fatty alcohol formation for WE biosynthesis in *M. aqualeolei* VT8 [40].

Other Gram negative marine bacterium with the ability to accumulate neutral lipids is the hydrocarbon-degrading *A. borkumensis* [12]. Interestingly, *A. borkumensis* SK2 possesses the

capability for producing more than 20% (of the CDW) of TAG plus minor amounts of WE. This feature differentiate strain SK2 from other Gram negative lipid-accumulating bacteria, such as *A. baylyi* ADP1 or *M. aqualeolei* VT8, which produce predominantely WE as storage lipids. *A. borkumensis* SK2 possesses two genes coding for WS/DGAT enzymes (called *atfA1* and *atfA2*), which exhibit activity with a broad range of substrates [12] (Table 1). Detailed molecular studies revealed that AtfA1 is the main contributor to TAG and WE accumulation in strain SK2, whereas AtfA2 is not indispensable for this process. When both *ws/dgat* genes were inactivated, cells were still able to produce low amounts of TAG (approx. 5–10% CDW), suggesting the presence of an alternative biosynthesis pathway [15]. Interestingly, *A. borkumensis* SK2 does not possess genes coding for fatty acyl- or fatty aldehyde reductase enzymes in its genome, as has been reported for other marine Gram negative lipid-accumulating bacteria, such as *A. baylyi* or *M. aqualeolei*. For this reason, strain SK2 is able to accumulate TAG but not WE from unrelated carbon sources such as pyruvate. WE are accumulated solely when cells are grown on the alkane hexadecane, which provides preformed precursors, such as fatty acyl and fatty alcohols, for WE biosynthesis.

Other interesting feature of *A. borkumensis* SK2 is its ability to export neutral lipids at the extracellular milieu; however the export mechanism present in this strain is still unknown [41]. The understanding of biological mechanisms of these bacteria for excreting lipids is of great importance for designing a cost-effective biotechnological production process. Cell harvest for lipid extraction is an expensive energy-intensive process; thus, the improved production of extracellular lipids could enable the optimization of the bioprocess.

6. Triacylglycerol- and wax ester-accumulating machinery in Gram positive actinobacteria

Actinobacteria usually accumulate TAG during cultivation of cells on diverse carbon sources under nitrogen limiting conditions, and in some cases when cells are grow on *n*-alkanes o *n*-alcohols, they also accumulate WE [8–10]. Despite recent advances in our understanding of neutral lipid metabolism occurred in the last years, the understanding of this process in actinobacteria is still fragmentary. Members of *Streptomyces*, *Mycobacterium* and *Rhodococcus* genera are the most investigated actinobacteria regarding the molecular biology and genetics of TAG accumulation.

6.1. *Streptomyces*

Rodriguez et al. [42] demostrated that *S. coelicolor* A3(2) possesses two genes coding for malic enzymes (decarboxylating malate dehydrogenases), which are involved in the biosynthesis of TAG and the polyketide antibiotic actinorhodin (Table 1). SCO2951 and SCO5261 encode NAD^+ and $NADP^+$-dependent malic enzymes, respectively. The authors proposed that both enzymes participate in the carbon flux homeostasis in *S. coelicolor* rather than providing NADPH for lipid biosynthesis. Thus, reactions catalyzed by NAD^+ and $NADP^+$-dependent malic enzymes (SCO2951 and SCO5261) may be part of the metabolic map assumed by *S. coelicolor* for accumulating TAG. Recently, Comba et al. [43] investigated the role of phosphatidic acid phosphatase (PAP) enzymes in TAG accumulation by *S. coelicolor*. PAP enzyme catalyzes the dephosphorylation of phosphatidic acid producing DAG, the lipid precursor for TAG biosynthesis. This reaction belongs to the Kennedy pathway, the main TAG biosynthesis route in prokaryotes, which involves the sequential acylation of the *sn*-1,2 position of G3P, resulting in the formation of phosphatidic acid (Fig. 1). After the removal of the phosphate group catalyzed by PAP enzyme occurs the final acylation step of the resulting DAG to TAG. Thus, PAP enzyme catalyzes the reaction preceding that catalyzed by the WS/DGAT enzyme for TAG biosynthesis (Figs. 1 and 2). Two PAP enzymes, Lppα (SCO1102) and Lppβ (SCO1753), are involved in DAG production for TAG biosynthesis in *S. coelicolor* [43] (Table 1, Fig. 1). These enzymes seem to contribute to TAG biosynthesis in a concerted way, since the disruption of either, *lppα* and *lppβ* caused no alteration in TAG accumulation, whereas the inactivation of both genes produced a significant decrease of TAG content (between 40 and 65%). Interestingly, the overexpression of *lppα* and *lppβ* separately promoted an increase of TAG production (approximately of 15 and 29%, respectively) [43]. After a deep bioinformatic analysis, three genes (*sco0123*, *sco0958*, and *sco1280*) potentially encoding WS/DGAT enzymes were found in *S. coelicolor* [20]. Molecular and genetic *in vitro* and *in vivo* approaches confirmed that the acyltransferase coded by *sco0958* gene is directly involved in TAG biosynthesis and accumulation in this microorganism. SCO1280 seems to be an active WS/DGAT with minor contribution to TAG biosynthesis, whereas the activity of SCO0123 is not related to the synthesis of such lipids [20]. Interestingly, the triple mutant of *S. coelicolor* was still able to produce considerable amounts of TAG, suggesting the occurrence of alternative TAG biosynthesis pathways. *In vitro* approaches for meassuring DGAT, DAG:DAG acyltransferase and PDAT (phospholipid:DGAT) activities, revealed that *S. coelicolor* possesses alternative acyl-CoA-dependent acyltransferase and PDAT reactions that contribute, at least in part, to TAG biosynthesis in this bacterium [20]. On the other hand, Kaddor et al. [26] investigated the accumulation of neutral lipids in *S. avermitilis* MA-4680, which was able to produce TAG from glucose and TAG plus minor amounts of WE during cocultivation of cells on glucose and hexadecanol as carbon sources. The authors demostrated that strain MA-4680 accumulated TAG using the classical acyl-CoA dependent DGAT pathway mediated by a WS/DGAT coded by *sav7256* gene [26] (Table 1 and Fig. 1). Based on *in vitro* approaches, the authors proposed that SAV7256 is only in part responsible for TAG synthesis in *S. avermitilis*, and additional unidentified DGAT isoforms are probably present in cells. All these enzymes integrate the set of genes/proteins required for the *de novo* TAG biosynthesis pathways in *Streptomyces* members.

In other study, Arabolaza et al. [44] identified and characterized a novel transcriptional factor, called FasR (SCO2386), which controls the expression of fatty acid synthesis genes (FabDHPF operon) in *S. coelicolor*. The mutation of FasR produced severe growth defects and significant decrease of fatty acid synthase activity and TAG accumulation. The authors indicated that the reduction of TAG content in the mutant could be related to the limited availability of fatty acids [44]. Since this pathway provides fatty acids for TAG formation, and FasR governs the activation of fatty acid synthesis, this transcriptional factor could be considered as part of the TAG-accumulating machinery in *S. coelicolor*, at least when cells are grown on unrelated carbon sources. It would be interesting to analyze the dependence of TAG accumulation on FasR when exogenous free fatty acids are supplied into de culture media.

6.2. *Mycobacterium*

Several studies have been focused on molecular aspects of TAG biosynthesis in mycobacteria, because of the importance of such lipids during the infection by *M. tuberculosis* or *Mycobacterium bovis*. Mycobacteria produce TAG in the form of intracellular lipid droplets under various stress conditions, such as hypoxia, exposure to nitric oxide and acidic environments [45]. These conditions induce nonreplicating persistent cells, which are phenotypically drug-resistant [46,47]. During the infection, mycobacteria perturb cellular lipid homestasis of human macrophages, resulting in the formation of foamy macrophages (FM), which contain abundant lipid

bodies in the cytoplasm [48,49]. These lipid-rich FM within human granulomas provide a source of nutrients and energy for persistent *M. tuberculosis* [48,49]. In this context, mycobacteria import fatty acids derived from lipid bodies in the host to resynthesize TAG that are then stored as intracellular lipid inclusion bodies [50]. The acquisition of TAG by mycobacterial cells seems to operate via lipid hydrolysis into fatty acids, which are then imported and used for TAG resynthesis [48]. Mishra et al. [51] suggested that Lip Y (Rv3097c) is the main responsible for the hydrolysis of host lipids into fatty acids in cells of *M. tuberculosis*. The authors demonstrated that Lip Y is mainly present at the surface of cell wall in mycobacteria [51]. Low et al. [45] proposed that the use of TAG as endogenous carbon and energy source is essential for the regrowth of mycobacterial cells during their exit from the hypoxic nonreplicating state.

M. tuberculosis possesses 15 genes coding for WS/DGAT isoenzymes, whereas at least 12 WS/DGAT's occur in *M. bovis* [19,23]. In this context, the acyltransferase coded by *Rv3130c* gene (also known as *tgs1*) is the main active WS/DGAT involved in TAG biosynthesis in *M. tuberculosis* (Table 1). Deletion of *tgs1* resulted in a drastic decrease, but not a complete loss in TAG accumulation, suggesting that other isoenzymes also contribute to TAG synthesis by cells [50]. Interestingly, the acyltransferase Tgs1 is a member of DosR-controlled regulon in *M. tuberculosis*, which is upregulated under hypoxia during the infection [52,53]. In this context, Daniel et al. [54] have recently shown that the acyl-CoA synthetase named FACL6 modulates TAG accumulation when *M. tuberculosis* enters dormancy (Table 1, Fig. 2). A mutant strain lacking FACL6 exhibited a significant decrease in the accumulation of intracellular TAG by cells under dormancy-inducing conditions, whereas complementation partially recovered the wild type phenotype [54]. The authors proposed that FACL6 possibly plays an important role in the channeling of fatty acids into TAG. Thus, this protein seems to be involved in the activation of fatty acids that are imported by *M. tuberculosis* from host lipid sources (Table 1 and Fig. 2).

Low et al. [46] reported the identification of five novel proteins that were specifically associated with lipid droplets from hypoxic nonreplicating persisting *M. bovis* BCG. The authors generated single gene deletion mutants of four of them (*tgs1*, *tgs2*, *BCG1489c* and *BCG1169c*) and the respective complemented strains. Tgs1 and Tgs2 were abundant WS/DGAT proteins in the mycobacterial lipid droplets. The authors proposed that Tgs2 may possess a broader preference for FA substrates compared with that of Tgs1. *BCG1489c* coded for a putative 1-acylglycerol-3-phosphate acyltransferase (AGPAT), which catalyzes the formation of phosphatidic acid in the Kennedy pathway (Figs. 1 and 2). This enzyme may play a prominent role in TAG synthesis in mycobacteria, since the deletion of this gene resulted in a significant reduction of TAG levels in cells. BCG1169c was a protein with unknown identity unique to *Mycobacterium* family, which appears to play a role in TAG biogenesis. The deleted mutant of this gene exhibited a similar phenotype than the knockout mutant of *tgs1* [46]. The authors proposed that BCG1169c may be a co-activator or a regulator of enzymes involved in TAG synthesis; although its role remains to be determined. Finally, the *BCG1721* gene encoded a presumably bifunctional enzyme with lipase and potential long chain acyl-CoA synthase (ACSL) activities (Table 1). It was proposed that this protein is able to hydrolyze TAG via the lipase domain to produce free FA, which are next activated to acyl-CoA with the help of the ACSL domain. The resulting FA may be distributed to different pathways, such as β-oxidation, synthesis of phospholipids for membrane turnover, or reesterification to TAG [46]. The authors proposed that this protein may be essential for cell growth since repeated effort to generate a gene deletion mutant failed [46].

In other interesting work performed in *M. tuberculosis*, Elamin et al. [55] reported the involvement of the mycolyltransferase Ag85A enzyme, which usually participates in mycolic acid synthesis, in TAG formation (Table 1, Fig. 2). The authors demonstrated that the protein Ag85A exhibits DGAT activity in addition to the usual function as mycolyltransferase. Overexpression of Ag85A induced a qualitative and quantitative modulation of glycolipids and mycolic acids in cell wall, and an increase in TAG formation in *M. smegmatis* [55]. This study demonstrated the complexity of TAG metabolism in such microorganisms, and the existence of alternative acyltransferase reactions dealing with TAG biosynthesis in mycobacteria and probably in other mycolic acid-containing actinobacteria. Despite of the importance of neutral lipids in pathogen mycobacteria, the understanding of TAG metabolism in mycobacteria is still rather limited.

Bacon et al. [56] reported the synthesis of novel saturated and unsaturated WE by *M. tuberculosis* during cultivation of cells under iron-limited growth conditions. The location of WE in mycobacterial cells was not determined, but the authors proposed that the linear molecules of WE may interact with long chains of mycolic acids in the cell envelope. They postulated that WE were formed from fatty acids hydrolyzed from TAG mediated by Lip Y (Rv3097c), which can also catalyze the synthesis of WE [56]. Thus, the accumulation of the novel WE in this study was not attributed to the WS/DGAT class of enzyme in *M. tuberculosis.*

In other study, Sirakova et al. [57] reported the identification of two genes in *M. tuberculosis*, *Rv3391* (*fcr1*) and *Rv1543* (*fcr2*), which encode fatty acyl-CoA reductase enzymes that produce fatty alcohols from acyl-CoA for the generation of WE under *in vitro* dormancy-inducing conditions (Table 1). The authors postulated that WE accumulate in the cell wall of mycobacteria, to act as a permeability barrier limiting cell replication by inhibition of nutrient uptake, rather than as an endogenous carbon and energy reserve.

6.3. *Rhodococcus*

The first report on TAG biosynthesis and accumulation by a member of *Rhodococcus* genus was in a strain isolated from a soil sample at a gas work plant in Moringen (Germany), which was later identified as *R. opacus* PD630 [10]. Later, we demonstrated that TAG accumulation was a common feature among rhodococci [17,58]. Most of the advances in the basic knowledge on TAG biosynthetic machinery in rhodococci have been obtained using *R. opacus* PD630 and more recently *R. jostii* RHA1 as model microorganisms. Recently, different integrated "omics" studies were performed to unravel TAG biosynthesis in the oleaginous *R. opacus* PD630 and *R. jostii* RHA1 strains [27,34,59,60]. Such global works clearly demostrated that the massive biosynthesis and accumulation of TAG by rhodococci demands a complex and specific metabolic network involving several reactions at different levels of the metabolism. Holder et al. [59] proposed that 261 genes are implicated in the *R. opacus* PD630 TAG's cycle. Proteomic analyses performed in *R. jostii* RHA1 demonstrated that several changes at different levels of cellular metabolism must occur to generate an oleaginous physiological state; including the activation of PPP and ED pathways to produce precursors and NADPH, glycogen mobilization, activation of glyceroneogenesis for generating G3P, degradation of amino acids to produce acetyl-CoA, propionyl-CoA and NADPH, inhibition of L-ectoine biosynthesis which consumes acetyl-CoA and NADPH, and activation of specific enzymes/proteins involved in TAG biosynthesis and accumulation [34].

Rhodococci usually accumulate TAG but not WE during cultivation of cells on glucose or gluconate, which are degraded to acetyl-CoA as precursor of the *de novo* biosynthesis pathway [10,17]. In contrast, during cultivation with related substrates, such as hexadecane or hexadecanol, rhodococcal cells utilize the preformed fatty acids and fatty acyl alcohols derived from the oxidation of the substrates for TAG and WE biosynthesis. These

microorganisms seem to be unable to produce WE from unrelated carbon sources due to the absence of fatty acyl reductase genes/enzymes in their genomes. Thus, the ability of rhodococci to produce TAG and/or WE depends on which intermediates are present in cell metabolism, since their WS/DGAT enzymes can potentially synthesize both, TAG and/or WE (Figs. 1 and 2). Recently, Shields-Menard et al. [61] reported the identification of WE and TAG in lipid extracts obtained from *Rhodococcus rhodochrous* grown on glucose as sole carbon source. This result suggested that this strain poseesses the mechanism to produce the necessary fatty alcohol intermediates from unrelated substrates, such as glucose, for WE synthesis. However, the identification of WE in glucose-grown cells should be confirmed using a more accurate chemical method than thin layer chromatography.

The TAG biosynthetic machinery of oleaginous rhodococci may include enzymes involved in different reactions of metabolism, transporter proteins, structural components of lipid inclusions, and transcriptional regulators at different hierarchical levels (global and specific regulators). At this time, seven genes from the oleaginous *R. opacus* PD630 and *R. jostii* RHA1 strains directly related to TAG metabolism have been cloned and functionally characterized (Table 1). MacEachran and Sinskey [62] identified and characterized a gene (called *tadD*) coding for a non-phosphorylative glyceraldehyde dehydrogenase enzyme (GAPN) in *R. opacus* PD630, which catalyzes the reversible oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate with the production of NADPH, which can be used for lipid synthesis (Table 1, Fig. 2). The authors proposed that this NADPH-depending reaction is specifically activated during cultivation of cells under TAG accumulating-conditions, instead of the canonical reactions catalyzed by the phosphorylative glyceraldehyde dehydrogenase (GapA) and phosphoglycerate kinase, which oxidizes glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate, while reducing NAD^+ to NADH, and converts the last intermediate to 3-phosphoglycerate, yielding one molecule of ATP [62]. It was hypothetized that during vegetative growth phase, cells metabolize glucose via the standard glycolytic pathway using the phosphorylative GapA, whereas during TAG accumulation cells switch to the non-phosphorylative glycolitic pathway mediated by TadD (GAPN). Results of molecular assays were consistent with this hypothesis: overexpression of GapA in strain PD630 produced a decrease of TAG content, and TadD overexpression resulted in an increase of such lipids in cells [62]. Interestingly, proteome studies performed in *R. jostii* RHA1 during TAG accumulation showed significant increases in the abundance of three aldehyde dehydrogenases containing the conserved motifs typical for a GAPN [34]. Since results of this study demonstrated that TadD protein plays an enzymatic role in TAG biosynthesis and accumulation in *R. opacus* PD630, we can considered this enzyme as part of the TAG biosynthetic machinery in rhodococci (Table 1, Fig. 2).

It is known that lipid transporters actively participate in the maintenance of lipid homeostasis in eukaryotic cells [63,64]. In this context, lipid transporters may play a similar role in TAG-accumulating rhodococci. Recently, we identified an ATP-binding cassette transporter in *R. jostii* RHA1 (called Ltp1) as an importer of long-chain fatty acids [65]. Our results suggested that Ltp1 plays a role in lipid homeostasis principally during cell growth, probably modulating fatty acid recycling during membrane remodeling and the distribution of fatty acids between different metabolic pathways and lipid species. The abundance of Ltp1 was significantly higher during vegetative growth phase in comparison to cells during TAG-accumulating conditions [34]. In addition, *ltp1* seems to be essential for survival of strain RHA1, since repeated efforts to generate a gene deletion mutant failed [65]. Interestingly, *ltp1* was clustered with other genes coding for the three putative acyltransferase enzymes of the Kennedy pathway for TAG biosynthesis, including one WS/DGAT. Orthologues of *ltp1* was uniquely present in those species considered as oleaginous rhodococci, such as *R. opacus* and *R. jostii*. Overexpression of *ltp1* in the RHA1 produced a significant increase of TAG formation. All these results suggest that Ltp1 is a component of the dynamic network designed to mantain lipid homeostasis in oleaginous rhodococci. TAG biosynthesis seems to be one of the metabolic pathways that accept fatty acids from this lipid transporter in strain RHA1. In this context, TAG stores may buffer the intracellular availability of long-chain fatty acids limiting their accumulation, since they are physiological active and potentially toxic intermediates [66] (Table 1, Fig. 2).

Other studies identified and characterized different genes coding for key enzymes of Kennedy pathway directly involved in TAG biosynthesis in *R. opacus* PD630 as well as in *R. jostii* RHA1. Two genes encoding WS/DGAT enzymes (called *atf1* and *atf2*) were cloned and characterized in detail in *R. opacus* PD630, which possesses at least 17 putative homologous proteins in its genome [21,22]. The contributions of *atf1* and *atf2* to TAG accumulation by strain PD630 were significant: the deletion of the respective genes resulted in a significant decrease of TAG biosynthesis and accumulation, and their overexpression promoted an increase in TAG content in cells [21,22]. The orthologue gene of *atf2* in *R. jostii* RHA1 (*RHA1_RS07790*) was highly synthesized during cultivation of cells under TAG accumulating conditions, confirming the role of this WS/DGAT isoenzyme in TAG biosynthesis by oleaginous rhodococci [34]. However, additional still unknown acyltransferases are contributing to TAG biosynthesis in strain PD630 and probably also in RHA1, because the respective *atf1* and *atf2* single mutants, and the double *atf1* and *atf2* mutant strain of PD630 still accumulate significant amounts of TAG [22].

Hernández et al. [67] identified a gene coding for a phosphatidic acid phosphatase type 2 enzyme (PAP2) from *R. jostii* RHA1, which produces DAG for TAG biosynthesis in the Kennedy pathway (Table 1, Figs. 1 and 2). Oleaginous rhodococci possess a higher abundance of genes encoding putative PAP enzymes than other rhodococcal species. The high diversity and abundance of PAP-like enzymes in TAG-accumulating rhodococcal specialists may permit cells to form different DAG pools for the selective formation of phospholipids or TAG, and/or may permit cells the independent regulation of isoenzymes at both, transcriptional and enzymatic levels. The protein product of *RHA1_RS00400* gene of strain RHA1 is a membrane-associated lipid phosphatase enzyme with the ability to produce DAG from intracellular phosphatidic acid [67]. The role of this PAP2 seems to be relevant not only for TAG synthesis, but also for cell growth since only when extrachromosomal copies of *RHA1_RS00400* gene were present in the wild type strain, the chromosomal deletion of the gene occurred. Altogether, RHA1_RS00400 is other component of the TAG biosynthetic machinery of *R. jostii* RHA1 feeding DAG intermediates directly to TAG synthesis (Table 1, Figs. 1 and 2). This enzyme may also participate in the control of DAG availability in such cells, considering that DAG are physiological active and eventually toxic intermediates; thus, their concentration in cells must be finely controlled.

The synthesis and accumulation of TAG should be finely coordinated with the lipid body assembly in oleaginous rhodococci, which permit cell accomodating and stabilizing the hydrophobic storage lipids in the aqueous cytoplasm of cells. For this reason, lipid droplets should possess an ordered structure, which may involve different components, such as structural proteins and phospholipids, among others. In this context, MacEachran et al. [68] identified and characterized a lipid body-associated protein (called TadA) with a structural role in *R. opacus* PD630 (Table 1). After screening 5000 random PD630 mutants using a transposon mutagenesis approach, the authors found a mutant strain which accumulated 30–40% less TAG, and formed slightly larger lipid bodies at early time points in comparison to the wild type. Complementation of the

mutant with copies of *tadA* gene produced an increase in TAG content, whereas its overexpression in the wild type strain resulted in the formation of very large lipid bodies. Finally, the authors of this study demonstrated that TadA protein is localized in the lipid bodies in *R. opacus* PD630 [68]. In other study, Ding et al. [69] obtained highly purified lipid bodies from *R. jostii* RHA1 and characterized the lipid body proteome. They identified a major lipid body-associated protein coded by *RHA1_RS10270* gene, which is the homolog of TadA from *R. opacus* PD630. Similar to TadA mutant, the deletion of *RHA1_RS10270* gene induced the formation of supersized lipid bodies [69]. In this context, the protein encoded by *RHA1_RS10270* gene was one of the three most abundant proteins during TAG accumulation, as recently revealed a proteome analysis applied to *R. jostii* RHA1 [34]. On the other hand, Chen et al. [60] conducted an integrated omics study including complete genome and transcriptome analyses, and a proteome of isolated lipid bodies from strain PD630. Integrating these three omics resulted in the identification of 177 proteins involved in lipid metabolism and lipid body dynamics. Among several enzymes and proteins, the authors identified four dynamin (LPD02043, LPD02044, LPD02062 and LPD02063) and three SNARE-like proteins (LPD02118, LPD02119 and LPD03976) which may be involved in lipid body dynamics [60]. Dynamin and SNARE-like proteins of *R. opacus* PD630 may play similar functions in the lipid body dynamics as in eukaryotes, in which play important roles in lipid body budding and fusion process. Among all identified proteins, a structure-like protein (LPD06283) was deleted for confirming its function in PD630 lipid body structure. Lipid inclusions of the deleted mutant were significantly larger in comparison to the wild type [60]. LPD06283 is identical with strain PD630 TadA and its orthologue (RHA1_RS10270) from *R. jostii* RHA1.

The specific metabolic state assumed by rhodococci during TAG accumulation seems to require the substantial rearrangement of metabolic network probably involving hundred of individual reactions that are simultaneously taking place in the cell, in order to synthesize all the metabolic components that are needed for supporting production of high amounts of fatty acids and TAG. The integration of results derived from global studies and those from experimental approaches focused on specific genes/proteins may permit the understanding of how microorganisms are able to synthesize and accumulate so much fatty acids and TAG. In general, all microorganisms are able to synthesize minimal amounts of lipids for their membranes and other functions, but only a relatively small number of bacteria are able to accumulate high amounts of lipids (more than 20% of cellular dry weight). Thus, the biosynthetic machinery of lipids is common to all microrganisms; however, there must be relevant physiological and metabolic differences between the oleaginous and non-oleaginous microorganisms. Oleaginous rhodococcal strains may have evolved a dynamic metabolic network designed to maintain a high carbon flux for massive fatty acids and TAG synthesis after growth is arrested. In general, oleagenicity might be based on the ability of cell to produce a continuous supply of acetyl-CoA and sufficient NADPH, as the necessary precursor and reductant for fatty acid biosynthesis. Moreover, cells must possess efficient mechanisms for controlling the availability of key intermediates, such as G3P, acyl-CoA's, phosphatidic acid and DAG [66]. A carefully balance must be maintained by oleaginous rhodococcal cells between buffering the potential toxic or phsyiologically active intermediates while ensuring adequate supply of the necessary energy, reducing equivalents, and precursors for TAG synthesis and accumulation. The cellular pools of the key intermediates G3P, acyl-CoA, phosphatidic acid and DAG are surely dynamic populations that have to be tightly regulated in cells. This situation demands the interaction between fatty acid uptake, TAG synthesis and lipolysis, which may be tighly coupled. In addition, TAG synthesis may be also coupled to changes in phospholipid synthesis, since these processes share common metabolic pathways (Fig. 2). For instance, phosphatidic acid phosphatase enzyme may control the balance between phosphatidic acid and DAG utilization by the CDP-DAG and the Kennedy pathway, as has been reported for eukaryotes [70]. Other example could be the simultaneous upregulation of the typically G3P degrading enzyme GlpD and the typically G3P synthesized enzyme GpsA, which seem to work together for the control of NADPH and G3P availability in cells of the oleaginous *R. jostii* RHA1 during TAG accumulation [34]. Altogether, all these genes/enzymes dealing with the control of key intermediates may contribute with the metabolic map established by rhodococcal cells during lipid accumulation. Oleaginous rhodococcal lipid models, such as *R. opacus* PD630 and *R. jostii* RHA, possess large genomes (approx. 9 Mb) with a large expansion in homologous genes involved in fatty acids and TAG biosynthesis and degradation [27,59,71]. The high redundancy of genes coding for isoenzymes in their genomes may determine the occurrence of an "oleaginicity gene set" of enzymes which are specifically activated when cells are accumulating storage lipids. All these genes/proteins may be components of the TAG-accumulating machinery evolved by these microorganisms during the evolution. In addition to metabolic enzymes and structural lipid body proteins, these components include the participation of an unknown regulatory circuit probably integrated by global as well as specific regulatory proteins, which finely coordinate the shift from the vegetative cellular state to the storage status. In this context, the mechanisms that control the regulation of lipid metabolism genes in oleaginous rhodococci, which are more relevant for TAG accumulation, are largely unknown.

6.4. Comparison between Rhodococcus and Mycobacterium genera

Rhodococcus and *Mycobacterium* are closely related genera which may share biochemical characteristics of genes and enzymes that are involved in lipogenesis and lipolysis. It can be assumed that the basic pathways of fatty acid and TAG metabolism are analogous for both genera. These microorganisms differ radically from other bacteria in their high content of lipids found in their different cellular structures. A large portion of their genomes codes for enzymes involved in lipid metabolism [59,71,72]. The Kennedy pathway seems to be the main route for the synthesis of TAG in both bacteria. Some genes/enzymes of this pathway have been identified and characterized in rhodococci and mycobacteria, such as AGPAT, PAP2 and WS/DGATs (i.e. Tgs2 of *M. bovis* BCG shares 57% of identity with Atf2 from from *R. opacus* PD630) (Table 1). In general, members of these genera possess high redundancy of lipid metabolism-related genes; thus, it is necessary to determine which of these putative isoenzymes are more relevant for TAG metabolism in representative rhodococcal and mycobacterial strains. Since pathogenic mycobacteria utilize preformed fatty acids acquired from host lipids for the synthesis and accumulation of TAG, it is important to identify and characterize in detail the set of genes/proteins involved in the hydrolysis of external lipids, transport and cellular distribution of fatty acids, which may represent potential targets for interference and drug development. In this context, the identification of an ATP-binding cassette transporter in *R. jostii* RHA1 (Ltp1), which was an essential importer of long-chain fatty acids, could be relevant for mycobacteria with clinical importance [65]. We did not find orthologues of Ltp1 in mycobacteria; however, similar transporter proteins may be involved in the fatty acid import and distribution to lipid pathways in these bacteria, such as D806_5739 of *M. smegmatis* MKD8 (42% of identity) or Rv1272c of *M. tuberculosis* H37Rv (32% of identity). The advances on the basic knowledge of TAG metabolism in rhodococci can be useful for orientating research in mycobacteria.

7. Concluding remarks

It is now clear that the difference between a WE/TAG-accumulating bacterial strain; from other that is not able to produce such storage lipids, is not simply the presence/absence of key enzymes involved in their synthesis. WS/DGAT enzymes are certainly indispensable for storage lipid biosynthesis, but they must work within an integrated metabolic and regulatory network in lipid-accumulating bacteria. This metabolic network, which is dynamic and changing, can enable a cell to efficiently respond to changes in environmental conditions. In this context, a bacterial cell of native strains synthesizes WE/TAG at a reasonable appropriate amount that support survival of the cell in its natural environment. Metabolic networks of lipid-accumulating bacteria likely evolved to become more efficient. This could be the main reason why bacterial hosts such as *E. coli*, which is naturally unable to produce WE/TAG, is not able to synthesize substantial amounts of such lipids when heterologously expresses key genes from lipid-accumulating bacteria.

In general, Gram negative lipid-accumulating bacteria have a complete set of genes/proteins for supporting WE/TAG biosynthesis, but with a more simplified configuration for lipid metabolism, in comparison to oleaginous actinobacteria, such as *R. opacus* and *R. jostii*. Taking into account the fragmentary results available on bacteria able to accumulate neutral lipids, the WE/TAG-accumulating machinery in prokaryotes seems to include the following components: (i) Specific genes coding for WS/DGAT enzymes uniquely present in lipid-accumulating bacteria. (ii) Genes coding for proteins involved in the assembly, stabilization and structure of lipid inclusion bodies, only present in bacterial strains able to accumulate WE/TAG. (iii) Genes coding for different enzymes catalyzing common reactions occurring in both, lipid-accumulating and non-accumulating bacteria; but working within a different metabolic and regulatory network. Among these enzymes, we can consider some enzymes of the central metabolism (non-phosphorylative glyceraldehyde dehydrogenase), those involved in fatty acid biosynthesis (acetyl-CoA carboxylases, fatty acid synthases, etc), enzymes involved in the common pathway for TAG and phospholipid synthesis (1-acylglycerol-3-phosphate acyltransferase, phosphatidic acid phosphatase, etc), among others. (iv) Genes encoding transporter proteins which expose lipids to acceptor pathways, such as TAG biosynthesis. Lipid transporting proteins are probably required for maintaining a balanced distribution of lipids between lipid fractions in the cell, principally close to the intracellular face of membranes where WS/DGAT enzymes are usually located. (v) Genes encoding proteins involved in the fine regulation of the lipid accumulation process. The regulatory network of lipid-accumulating bacteria may include regulatory proteins which control the expression of specific genes of WE/TAG metabolism, and other global regulators which regulate a set of required reactions and processes at different hierarchical levels. Thus, oleagenicity in prokaryotes requires a special metabolic network involving concerted reactions and pathways, and a specific regulatory circuit for the fine control of cellular functions dealing with the appropriate distribution of metabolic intermediates that can result physiologically active and potentially toxic for the cell.

The combination of results obtained in model microorganisms using global studies with data from cells overexpressing diverse genes, as well as from cells lacking functional proteins upon mutation, will be indispensable to obtain a clearer picture of WE/TAG accumulation in prokaryotes.

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